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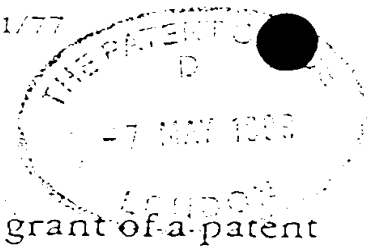

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ZENECA Limited
15 Stanhope Gate
London W1Y 6LN
UNITED KINGDOM

Patents ADP number *(if you know it)* — 6254007002

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

4. Title of the invention

CELLS AND ASSAY

5. Name of your agent *(if you have one)*

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Malcolm John HOUGHTON
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# Cells and Assay

The present invention relates to cells and particularly erythroid cells, to methods of producing them and their use in assays, as well as to vectors.

In animal cell expression, the ideal has always been to have a system capable of reproducible, high level, stable expression of the proteins of interest. The LCR/MEL expression system (Locus Control Region/Murine Erythroleukemia cells) complies with this demand as previously demonstrated by Amar *et al.*, (1995) J. Recept. Signal Tr. R. **15** 71-79; Egerton *et al.*, (1995) J. Mol. Endocrinol. **14** 179-189; Needham *et al.*, (1992) Nucleic Acids Res. **20** 997-1003, Needham *et al.*, (1995) Protein Expres. Purif. **6** 124-131; Newton et al., (1994) Protein Expres. Purif. **5** 449-457 and Shelton *et al.*, (1993) Receptor. Channel. **1** 25-37.

MEL cells are erythroid progenitor, robust, semi-adherent cells with a doubling time of only 10 to 16 hours, which are derived from spleens of susceptible mice infected with the Friend Virus Complex (Friend C (1957) J. Exp. Med. **105** 307-318). They are continuously dividing cells, arrested at the proerythroblast stage. Changes similar to normal red blood cell maturation can be induced with a variety of chemical agents, including polar-planar compounds like dimethyl sulfoxide (DMSO). This terminal differentiation causes an increase of globin gene expression which can result in α and β globin comprising up to 25 % of the total cell protein. The globin LCR enhancer is responsible for high levels of erythroid cell specific expression of globin proteins.

The human globin LCR has been utilised in the LCR/MEL system, alongside a human β-globin promoter *in cis*, to drive integration site position independent expression of cDNA and genomic sequences (Needham *et al.*, 1995 supra.). The globin LCR confers integration site independent expression on stably transfected genes which are linked *in cis* (Blom von Assendelft et al. (1989) Cell **56** 969-977; Talbot et al. (1989) Nature **338** 352-355). The human β-globin promoter and parts of the β-globin gene provide mRNA processing and maturation signals, give stability to the final mRNA and confer high expression levels in induced cells (Needham *et al.*, 1992 supra).

The LCR/MEL system has already been used to express a variety of proteins. Stable expression of electrophysiologically functional mammalian homo- and hetero-multimeric ion channel proteins has been obtained in MEL cells (Amar *et al.*, 1995 supra.; Shelton *et al.*, 1993 supra. and Monica Garcia-Alonso (1997) "Evaluation of the potential of Murine Erythroleukemia (MEL) cells as an expression system for nicotinic acetylcholine receptors" (Ph.D. thesis Reading University UK)). The LCR/MEL system is capable of producing functional secreted proteins (Needham *et al.*, 1992 supra.; Newton *et al.*, 1994 supra.). It has also been shown to produce very high levels of mammalian G-protein coupled receptors (sometimes known as seven-transmembrane helix receptors or 7TMR) as a source for ligand binding experiments (Egerton *et al.*, 1995 supra.; Needham *et al.*, 1995 supra.).

UK Patent No. 2251622 describes and claims expressions systems, including those based upon MEL cells, for the expression of heterologous polypeptides, in particular human proteins such as human growth hormone.

All of the previous examples of expression from MEL cells were of proteins of mammalian origin. High level expression of the genes was only seen after the recombinant MEL cell differentiation had been induced.

There are several approaches to achieve stable, heterologous expression of G protein-coupled receptors in animal cells (Vanden Broeck, 1996, Int. Rev. Cytol. 164, 189-268). The majority of examples have come from mammalian systems. In conventional mammalian cloning systems, it can prove to be labour intensive to produce stable recombinant cell lines reliably expressing large amounts of receptor as well as to produce large numbers of recombinant cells.

The LCR/MEL cell expression system resolves these problems, as it is capable of reproducible, high level, stable expression of receptors as well as being a robust semi-adherent cell line (Needham *et al.*, 1992). However, with the conventional LCR/MEL system, heterologous protein expression only occurs at high levels after induction of

differentiation of the cells into mature red blood cells. Unfortunately, this differentiation frequently leads to the loss of functionality of the signalling cascades usually linked to G protein-coupled receptors. This means that the existing LCR/MEL system is ideal for ligand binding assays on recombinant MEL cells, but cannot be used in functional assays ($Ca^{2+}$, IP3 or cAMP assays). As a consequence, functional assays with G-protein coupled receptors have to be performed in other systems.

According to the present invention there is provided an erythroid cell which is substantially undifferentiated but which expresses proteins under the control of a globin promoter thereof.

Such cells are particularly useful in that they allow functional as well as ligand binding assays to be effected depending upon whether or not they are induced prior to assay.

Suitable erythroid cells are murine erythroleukaemia (MEL) cells, rat erythroleukaemia cells (REL) and human erythroleukaemia cells (HEL), but are preferably murine erythroleukaemia cells.

Particular globin promoters which control expression of proteins in the cells of the invention are the β-globin promoters, such as human β-globin promoters.

Cells in accordance with the invention can be obtained by culture of uninduced erythroid cells for a sufficient period of time, usually over a period of a few months, until they become "leaky" in the sense that protein under the control of globin promoters are expressed.

Leakiness in cells can be detected by routine methods. For example, the cells can be screened for mRNA levels using for example Northern blotting techniques. Detection of protein mRNA, for example β-globin mRNA would be sufficient to indicate that the cells were in the correct stage.

Alternatively, the cells can be transformed with a reporter or marker gene which is placed under the control of a globin promoter, preferably a β-globin promoter, and detecting expression of the marker gene in uninduced cells. Suitable reporter or marker genes for use in this process are well known in the art and include for instance the β-galactosidase gene (β-Gal).

In a further alternative, it may be possible to determine that the cells are in the correct state because they are beginning to change colour, by taking on a pinkish hue, indicating that a progression to red differentiated erythroid cells has begun. This may be detected either spectrometically or by eye.

A yet further alternative is to detect expressed proteins themselves, for example using a conventional antibody type assay which may be either a direct or competitive assay. Examples of suitable proteins which may be detected include globins. Means of carrying out such assays are well known in the literature and include the use of labelled antibodies, for example radiolabelled or fluorescent antibodies, as well as enzyme-linked immunoassays (ELISAs).

A particular type of cell which can form cells of the invention are subclones of the MEL C-88 cell line, an example of which was deposited at the European Collection of Cell Cultures under the Accession number 99012801, deposited on 28 January 1999. This clone has been designated "MEL-C88L".

Cell lines of this type can be used in functional assays as illustrated hereinafter, since the cells retain nucleii which are lost or otherwise functionally silenced on terminal erythroid differentiation. Thus use can be made of the signalling pathways in the cell, such as those in which G-proteins are involved, where for example, globin promoters can drive the expression of heterologous proteins which normally functionally interact with a G-protein, in particular G-protein coupled receptor molecules (GPRC). These receptor molecules may be of mammalian or non-mammalian origin and in particular are insect receptors such as the *Locusta*

*migratoria* tyramine receptor (TyrLoc), or other receptors such as dopamine, octopamine, serotonin, or acetylcholine receptors such as muscarinic acetylcholine receptors.

In a further aspect, the invention provides a method for determining the interaction between a receptor protein and a potential agonist or antagonist therefor, said method comprising incubating a cell as defined above which has been transformed so that it expresses said receptor protein as a G-protein coupled receptor,
either (I) in (a) the presence and (b) the absence of said potential agonist; and/or
(II) in the presence of a known agonist and (a) the presence or (b) the absence of said potential antagonist; and
measuring and comparing G-protein induced signals in cells of (Ia) and (Ib) and/or (IIa) and (IIb).

The G-protein coupled receptor signal is induced in the presence of ligands for that receptor. In such a case, the G-protein coupled receptor which is expressed in MEL C88L cells, interacts with the G-protein and triggers a signalling cascade which may either increase or decrease the concentrations of various detectable components within the cell. Mechanisms by which these signalling cascade may operate are illustrated in the literature, for example, S. Klostermann et al., Perspectives in Neurobiology, (1996) 4, 237-252 and M.A.D. Fazia, FEBS Letters 410 (1997) 22-24.

The levels of these signals may be indicative of agonist or antagonist activity. This is particularly applicable if other possible target sites in the signalling cascade on which the compound may act have been eliminated, for example by carrying out specific assays for the other possible target sites, carrying out assays on untransformed cells, and/or using electrophysiological assays or studies on receptors or receptor preparations. Whether these changes are the result of the presence of the specific candidate agonist or antagonist can then be established by observing the difference between the signals generated by the cell line expressing the receptor protein of interest and the untransformed progenitor cell line i.e. cell lines differing only in the presence/absence of the target receptor. Where an elevated or heightened G-protein

coupled receptor induced signal results in an elevation of the amounts of a particular downstream component in the signalling cascade, the signal generated by (Ia) will be greater than that generated by (Ib) if the potential agonist is effective. Under similar circumstances, the signal from step IIa above will be lower than that obtained in IIb if the potential antagonist is effective. Conversely, if an elevated G-protein coupled receptor induced signal results in a decrease in the concentration of a particular downstream component in the signalling cascade, application of a potential agonist would result in a reduction of the levels of that component. This can often only be detected by amplifying the signal using a chemical which artificially stimulates the level of the component e.g. forskolin. Step (Ia) will then be lower than step (Ib). Where an antagonist in being assayed, the results of step (IIa) will be greater than (IIb), but can only be detected in the presence of forskolin.

In one embodiment, the G-protein coupled receptor induced signal is monitored by measuring the free calcium ion concentration of the cells. This can be done using known techniques, for example utilising a fluorescent indicator, such as fura-2 which binds free calcium ions, and whose fluorescent signal alters depending upon whether it is bound to calcium ions or free.

In an alternative embodiment, the G-protein coupled receptor induced signal is monitored by measuring the cyclicAMP (cAMP) levels within the cell, which may be increased or decreased, depending upon the nature of the G-protein coupled receptor. The G-protein coupled receptor induced signal activates the G-protein which interacts with adenylate cyclase enzyme either to increase or decrease the levels of cAMP in the cell. cAMP can be extracted from the cells and measured using commercially available kits such as scintillation proximity assay (SPA) kits available from Amersham International (UK).

The calcium signalling mechanism is similar in that the concentration of calcium ions found in cells changes (either increases or decreases) as a result of G-protein induced signal.

In yet a further embodiment, the cells may be further transformed with a reporter or marker gene, such as β-galactosidase, expression of which is regulated by the G-protein coupled receptor signalling cascade. Changes in the signal will then be apparent by detecting the expression levels of the marker gene.

If necessary, where the G-protein coupled receptor induced signal results in a decrease in the level of the measured cellular component, the changes in the signal can be amplified by adding chemicals which artificially stimulate the detectable chemical in the cell. For example, forskolin (FSK) is known to artificially stimulate cAMP levels in a cell by directly activating adenylate cyclase. Hence when the G-protein coupled receptor signal results in a decrease in cAMP levels, this decrease may appear more clearly if the agonist, with or without antagonist, is added together with forskolin. An effective agonist would be expected to reduce the amount of cAMP as compared to the forskolin alone. The presence of an effective antagonist would mean that the levels of cAMP would be higher than the test (IIb), in the absence of the antagonist.

In such cases, a further assay, in the presence of forskolin or the other artificial stimulant alone may be of assistance in the determining the levels of efficacy of the agonist or antagonist, and or in assessing the relative efficacies of various potential agonists and antagonists.

In all cases, cells of the invention are first transformed so that they express the G-protein coupled receptor of interest and clones which provide a good G-protein coupled receptor induced signal can be selected by testing each clone by adding varying amounts of the known receptor ligand, and if necessary, a chemical which stimulates the detectable cellular component (as discussed above). Clones which provide the most clearly distinguishable signals as between (Ia) and (Ib) and/or (IIa) and (IIb) above are selected for use in assays.

Cells of the invention can be used to express heterologous proteins, including human proteins. In particular however, the applicants have found that they are useful in the expression of non-mammalian and especially insect proteins.

To date, MEL cells giving a significant background level of G-protein coupled receptor expression in the absence of inducers such as dimethylsulphoxide (DMSO), have been used in a number of independent transformation experiments. In each case, even where a small number of transformed clones, for example ≥3, were analysed, isolates showing both efficient inducible but low level "leaky" expression of the introduced heterologous genes were identified.

The applicants have, for the first time, expressed an insect G-protein coupled receptor in the LCR/MEL system: the locust tyramine receptor, TyrLoc. This receptor was previously expressed in stably transfected *Drosophila* S2 cells (Vanden Broeck et al. (1995) J. Neurochem. **64** 2387-2395). The present results with the MEL-TyrLoc cells indicate that the pharmacology of the receptor expressed in these mammalian cells is similar to that in S2-TyrLoc cells. Both $Ca^{2+}$ and cAMP measurements demonstrate that there is a very efficient coupling of the expressed insect receptor to the endogenous, mammalian G proteins. This observation indicates that the use of this novel MEL expression system should not necessarily be restricted to the characterization and functional analysis of mammalian receptor proteins. Moreover, the ability of the LCR/β-globin promoter combination to confer high levels of expression, in a reproducible and position independent manner, is not affected in this MEL C88L cell clone.

This was shown by the fact that the monophenolic amine, tyramine (TA) is a much better agonist than octopamine (OA). It activates this receptor at concentrations which are 3-4 orders of magnitude lower than OA. Also, yohimbine proved to be a better receptor antagonist than chlorpromazine and mianserin.

These results clearly confirm the ligand binding data and the attenuating effect of TA on forskolin stimulated cAMP production which were previously obtained with Tyr*Loc* expressing S2 cells (Vanden Broeck *et al.*, 1995 supra.). G protein-coupled receptors for phenolamines (TA and/or OA) have been identified in other insect species (*Drosophila melanogaster:* Arakawa et al. (1990) Neuron **2** 343-354 and

Saudou et al., (1990) EMBO J. **9** 3611-3617; *Heliothis virescens*: Von Nickisch-Rosenegk et al., (1996) Insect Biochem. Molec. Biol. **26** 817-827) and in the mollusc *Lymnea stagnalis* (Gerhardt CC et al., (1997) Mol. Pharmacol. **51** 293-300). The *H. virescens* (K50*Hel*) and *L. stagnalis* (*Lym*-OA1) receptors are preferentially activated by OA, whereas the fruitfly receptor (Tyr/Oct-*Dro*) produces agonist-specific (TA versus OA) coupling to different second messenger systems when it is expressed in NIH 3T3 (Saudou *et al.*, 1990 supra. ), in CHO cells (Robb (1994) EMBO J. **13** 1325-1330) or in *Xenopus* oocytes (Reale et al. (1997) Brain Res. **769** 309-320).

Assays using the cells of the invention and methods in accordance with the invention have been shown to be effective in determining the relative strengths of receptor agonists and antagonists. Specifically, it has been found that the locust receptor Tyr*Loc* is more sensitive to TA than OA as agonists for both cAMP inhibition and $Ca^{2+}$ stimulation in MEL-TyrLoc cells.

These assays are useful in investigations into the biological function of molecules. For example, it is known that TA is the biosynthetic precursor of OA and it is present in many parts of the locust nervous system. The results reported here also imply that TA might be a very important neuro-active substance and this idea is strongly supported by the discovery of separate activities, binding sites and uptake systems for tyramine and octopamine in the locust central nervous system (Roeder T (1994) Comp. Biochem. Physiol. **107C** 1-12; Hiripi L et al., (1994) Brain Res. **633** 119-126.,; Downer et al., (1993) Neurochem. Res. **18** 1245-1248).

After induction, this clone also differentiates along the erythroid pathway and as a result boosts the expression levels of heterologous TyrLoc receptor proteins at least three- to four-fold. As a consequence, when induced, this clone loses the functionality of its signal transduction pathway, but is now ideal for ligand binding assays.

Thus the invention further provides an assay for detecting binding between a protein and a potential binding partner therefore, said method comprising (a) transforming a cell as described above so that the protein is expressed under the control of a globin

promoter, (b) detecting binding between said potential binding partner and the protein on the membrane of the cell. Optionally, the cells are induced after step (a) and prior to step (b), so as to obtain high levels of protein expression from fully differentiated cells.

Step (b) may be effected on whole cells, or on isolated membranes extracted from lysed cells. Suitably, the protein is a receptor and the potential binding partner is a ligand therefore. However, binding between other types of protein, such as naturally occuring proteins, antigens, immunoglobulins such as antibodies, and binding partners, in particular specific binding partners can be tested in this manner.

This work demonstrates that the MEL cell line can be an even more versatile system than previously thought. It may be used in a variety of situations, from functional (G-protein coupled receptor signalling cascade or ligand gated ion channels) to ligand binding assays, for both mammalian and insect, secreted or transmembrane proteins.

Vectors used in tranformation of the cell lines form a further aspect of the invention.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1a illustrates the plasmid used to insert the locust tyramine receptor (TyrLoc) into the multiple cloning site of pEV3, such that the globin LCR enhancer and the murine β-globin promoter drive integration site independent expression of the receptor cDNA;

Figure 1b illustates a reporter vector p3XVIP-hyg(P) which comprises three copies of a cAMP response element (3XVIP) upstream a minimal β-globin promoter arranged to drive the expression of a β-galactosidase reporter gene in the presence of cAMP in the cell;

Figure 2 is a graph showing the dose-response relationship between tyramine (TA) concentration and Ca2+ increase in MEL-TyrLoc cells compared to MEL C88L control cells which do not express the receptor;

Figure 3 is a graph showing a comparison of calcium responses in MEL-TyrLoc cells elicited by tyramine (TA) (10 nM) and octopamine (OA) (0.5 μM);

Figure 4 is a graph illustrating that the addition of different concentrations of yohimbin (Y) results in an inhibition of the response to 0.5 μM TA, but could not inhibit the response to high TA concentrations (5 μM);

Figure 5 is a graph showing control cAMP measurements of untransformed MEL-C88L cells in the presence of forskolin (FSK);

Figure 6 illustrates the inhibition of forskolin induced increase of cAMP by tyramine in MEL-TyrLoc cells; and

Figure 7 illustrates the results obtained with a β-gal assay using MEL cells transformed with a dopamine receptor and the reporter vector, where in each group, the first column indicates cells incubated in medium alone, the second shows those incubated with 30μM forskolin, the third colum shows the results of incubation with 10μM dopamine.

Example 1

Generation of the pEV3TyrLoc expression vector

The tyramine receptor cDNA from the locust *Locusta migratoria* was cloned into pEV3, downstream of the human globin locus control region (LCR) between the promoter and the second intron of the β-globin gene (Figure 1a) as follows.

The coding region of the TyrLoc cDNA was amplified by polymerase chain reaction (PCR) from pVJ12 and pVJ12-IEG (J. Vanden Broek et al., J. Neurochemistry (1995) 64, 6, 2387-2395) using the following oligonucleotide primers:

5' PCR primer (TyrLocF2):

5'-TTTTAAGCTTGAATTCAGATCTGCCACCATGAACGGGTCTTCGGCTGC-3'

(SEQ ID NO 1)

3' PCR primer (TyrLocRev):

5'-TTTTGGATCCGCGGCCGCGTCGACTCATGTCTTGAAGTGGAGCAGC-3'

(SEQ ID NO 2)

The 5' primer contains the restriction sites *Hind* III, *EcoR* I *and Bgl* II, and the consensus translation enhancing sequence (GCCACC) (M. Kozak, J. Mol. Biol. (1987) 196 (4) 947-450). The 3' primer contains the restriction sites *BamH* I, *Not* I *and Sal* I. A PCR product was obtained with *Pfu* Polymerase (Stratagene) utilising the manufacturers protocol.

The following PCR conditions were used: 1 cycle of denaturation at 96°C for 2 minutes followed by 40 cycles of denaturation at 96°C for 1 min, annealing at 58°C for 45 s and extension at 72°C for 3 min; followed by a final extension reaction of 10 min at 72°C . The resulting PCR product was cloned into the pCR-Script (Amp SK+) vector (Stratagene) using the manufacturers protocol. Sequence analysis of a clone, confirmed the presence of a correctly edited insert. This insert was released from the pCR-Script (Amp SK+) background using *EcoR* I and *Sal* I enzymes (Pharmacia Biotech Products) and cloned by standard techniques into pEV3 digested with the same pair of enzymes (Figure 1a).

The identity of the DNA utilised for MEL cell transformation was confirmed by restriction digestion and agarose gel electrophoresis and sequencing.

Example 2

Cell culture and cell transfections

Murine erythroleukemia C88 cells (Deisseroth A Hendrick D (1978) Cell **15** 55-63) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% foetal bovine serum and 2mM glutamine at 37°C, under 10% CO2 - 90% air. "Leaky" MEL cells, C88L, which allow low level uninduced expression of

globin genes in undifferentiated cells, were generated by prolonged culture of the cells (several months), prior to transfection studies.

The expression construct pEV3TyrLoc was introduced into leaky MEL-C88L cells by electroporation. Prior to transfection, 50 µg of pEV3TyrLoc were linearised at the unique *Asp* 718 site upstream of the neomycin cassette and downstream of the tyramine expression cassette. Transfection into the cell line MEL-C88L was performed by electroporation as described (Antoniou M (1991) "Induction of erythroid-specific expression in Murine Erythroleukemia (MEL) cell lines" *in* Methods in Molecular Biology Vol 7 Gene Transfer and Expression Protocols (eds Murray EJ) 421-434 The Humana Press Inc.).

After transfection, cells were diluted in culture medium to concentrations of about $10^4$, $10^5$ and $2 \times 10^5$ cells per ml and 1 ml aliquots were transferred to each well of a 24-well tissue culture plate (Gibco BRL-NUNC nunclon multidishes (polystyrene, radiation sterilised, with lids) 24 well plates, Cat # 143982A). Twenty four hours after the transfection, G418 was added to a concentration of 1 mg/ml in order to select for stable transfectants. Individual clones were picked, or pooled to generate populations, 7 to 10 days after the addition of selective medium.

For RNA purification and functionality studies, cells were maintained in exponential growth by passaging them every day for a period of 4 days (cells should be growing from $2.10^5$ cells/ml to $6\text{-}8.10^5$ cells/ml in 24 hours when in log phase). For the RNA purification, one half of the cells was induced using 2% DMSO and incubated for a further period of 4 days.

Example 3

RNA analysis

Following induction with 2% DMSO, a Northern blot was performed on RNA extracted from induced and uninduced clones and populations. Approximately $1.10^7$ cells were washed with phosphate-buffered saline and resuspended in 1 ml of RNAzol

B (Biogenesis). RNA was then purified according to the manufacturer's protocol. The RNA concentration was calculated from spectrophotometer readings at 260 nm.

Electrophoresis of the RNA samples (10 µg per lane) was performed through agarose gels containing 2.2 M formaldehyde in duplicate. The RNA was then transferred to a nylon membrane (Hybond-N, Amersham) in 20 x SSC. After transfer, RNA was covalently cross-linked to the membrane by short-wave ultraviolet irradiation using a U.V. Stratalinker™ 2400 (Stratagene). Each duplicate membrane was prehybridised and hybridised (Church GM & Gilbert W (1984) Proc. Natl. Acad. Sci. USA **81** 1991-1995; Feinberg AP and Vogelstein B (1983) Anal. Biochem. **132** 6-13 (Addendum: Anal. Biochem. (1984) **137** 266-267) using either $^{32}$P-labeled β-globin or $^{32}$P-TyrLoc-receptor probes.

There was a large increase in the production of TyrLoc mRNA after induction. Only clones that gave a strong signal on the Northern blot were used in further experiments.

Example 4

Ca2+ measurements

Elevations in intracellular $Ca^{2+}$ in response to stimulation with ligands of the tyramine receptor were measured as follows. Since MEL cells loose the ability to signal on differentiation, and as receptor expression was readily detectable without induction of the MEL cells, uninduced MEL C88L cells of the invention were used.

Ca2+ concentration was measured by using the acetoxymethyl (AM) ester of the fluorescent indicator fura-2 (Grynkiewicz G et al. (1985) J. Biol. Chem. **260** 3440-3450). Cells were washed with NCF buffer (135 mM NaCl, 5 mM KCl, 6 mM glucose, 0.62 mM MgCl2.6H2O, 10 mM HEPES, pH 7.4 with 4 mM CaCl2) and resuspended at a concentration of $2.10^6$ cells/ml in NCF buffer containing 2 µM fura-2-AM (Molecular Probes). After 1 hour incubation in the dark at 27°C, the cell suspension was subjected to centrifugation at 1200 rpm for 5 minutes, resuspended in an equal volume of NCF buffer and incubated for an additional 30 min in the dark. Aliquots were centrifuged as above and resuspended in 3 ml NCF buffer.

Measurements of intracellular calcium were made fluorimetrically in a LS-50B Luminescence Spectrophotometer (Perkin-Elmer) in the presence and absence of the appropriate test chemical. Excitation wavelength alternated between 340 and 380 nm. The fluorescence intensity was monitored at an emission wavelength of 510 nm.

The different clones were tested in order to see whether there was a difference in their functional response to 10 μM tyramine (TA), an endogenous insect neurotransmitter. All of the clones tested gave a similar response (data not shown), therefore only one clone was used in further experiments. Figure 2 shows a dose-response experiment for this clone: there was a clear transient increase in Ca2+ after addition of TA. The lowest concentration of TA tested was 0.01 μM. A maximal response was obtained when using 1 μM TA. Untransformed MEL-C88L cells gave no response with concentrations as high as 100 μM TA.

Addition of ionomycin (2μM), a $Ca^{2+}$-ionophore, to the cells resulted in a large increase of the intracellular $Ca^{2+}$ concentration, showing that the cells were properly loaded with fura-2. The $Ca^{2+}$ dependency of the TA response was tested by using NCF buffers with different $Ca^{2+}$ concentrations. In low $Ca^{2+}$ buffer (no extra $Ca^{2+}$ added to the buffer), the elevated $Ca^{2+}$ level quickly dropped down to its initial concentration. Also, the $Ca^{2+}$ release seen in the presence of low $Ca^{2+}$ buffer was greater than the release seen in high $Ca^{2+}$ buffer (4 mM $Ca^{2+}$) (data not shown).

These observations and results are consistent with the expectation that the initial cytoplasmic $Ca^{2+}$ increase comes from the release of the internal stores, whilst a sustained $Ca^{2+}$ level is achieved by an influx of $Ca^{2+}$ from the outside of the cell. The cytosolic $Ca^{2+}$ concentrations are probably lower in low $Ca^{2+}$ buffer due to establishment of an equilibrium across the cell membrane. This is the reason why the fura-2 340nm/380nm fluorescence ratio increases more in low $Ca^{2+}$ buffer, *i.e.* there was initially more free fura-2 compared to cells in high $Ca^{2+}$ buffer.

The effect of different agonists and antagonists of the tyramine receptor was investigated. The response to octopamine (OA) was measured, since OA is a

phenolamine which is structurally related to TA. Though it was used at a 50 times higher concentration, OA increased the Ca2+ concentration to a much lesser extent than TA, showing the specificity of the receptor for TA (Figure 3).

At concentrations below 20 μM, metaclopramide had no detectable effect on basal Ca2+ concentrations or on the response of the cells to TA. Chlorpromazine and mianserin clearly inhibited the Ca2+ increase induced by TA. Both naphazoline and tolazoline were weak antagonists (data not shown).

Yohimbine proved to be the strongest antagonist of the substances which were tested and a dose-response assay was performed (Figure 4). Yohimbine was added after 50 s in different concentrations (0.1, 1 and 2.5 μM). Subsequently, 0.5 μM and 5 μM of TA was added after respectively 150 and 350 s. Yohimbine produced a concentration-dependent inhibition of the response to 0.5 μM TA, but it could not inhibit the 5 μM TA response, within the concentration range employed.

Example 5

cAMP measurements

The effect of TA on the cAMP level of the transformed and untransformed cells was investigated. MEL-C88 and MEL-TyrLoc cells were seeded in 6-well plates to a density of $8.10^5$ cells/well and allowed to attach. The cell medium was removed and, in different wells, replaced with different NCF buffer solutions as follows:

(i) NCF + 1 μM tyramine (TA),
(ii) NCF + 10 μM forskolin (Fsk),
(iii) NCF + 10 μM Fsk + 1 nM to 10 μM TA.

where NCF comprised 135mM NaCl, 5mM KCl, 6mM glucose, 0.62mM $MgCl_2$, 10mM Hepes pH 7.4 4mM $CaCl_2$.

For the use of other ligands than TA, the solutions were:
(iv) NCF + 10 μM Fsk + 1 μM OA,

(v) NCF + 10 μM Fsk + 0.1 μM TA + 100 nM to 1 nM yohimbine,
(vi) NCF + 10 μM Fsk + 0.1 μM TA + 10 nM mianserin; and
(vii) NCF + 10 μM Fsk + 0.1 μM TA + 10 nM chlorpromazine.

All solutions contained 200 μM isobutylmethylxanthine (IBMX) in order to inhibit cAMP phosphodiesterase. In each study, triplicate wells were incubated with the same solution at 37°C for exactly 30 min.

In order to extract the cAMP, 100 % ice-cold ethanol was added to each well to a final concentration of 65 %. After 5 min incubation at room temperature, the solution was removed from the wells which were then rinsed with 65% ethanol. The eluates from the same well were then pooled together and evaporated using a speedvac. cAMP was then measured using the Scintillation Proximity Assay (Amersham), according to the manufacturer's recommended procedure. This experiment was done in triplicate.

Figure 5 shows the cAMP levels in untransformed MEL-C88L cells: addition of 1 μM TA did not result in any effect. Forskolin, with or without TA, increased the cAMP level more than 6 times.

Figure 6 shows the effect of TA on MEL-TyrLoc cells: TA significantly inhibits the forskolin-induced increase of cAMP. This response was dose-dependent; a minimal and maximal response was achieved when using 1 nM TA and 1 μM TA respectively. There was a 4-fold decrease in cAMP production with 1 μM TA when compared to 1μM OA, therefore, indicating that TA is a much stronger inhibitor of cAMP production in these cells.

Yohimbine inhibited the effect of TA in a dose dependent manner. The antagonistic effects of mianserin and chlorpromazine were also confirmed (data not shown).

The measurements were found to be highly reproducible.

Example 6

β-galactosidase assay using dopamine clones

MEL C88L cells were transformed with the serotonin and dopamine receptors using conventional methods and using northern blot analysis, 5-6 clones were chosen for further evaluation.

Generation of pEV3D-Dop1 Expression Vector

The Dopamine Receptor cDNA from the fruit fly Drosophila melanogaster was cloned into pEV3, downstream of the human globin locus control region (LCR) between the promoter and the second intron of the β-globin gene (Figure 1a) as follows.

The coding region of the D-Dop1 cDNA was amplified by PCR from pDMdop1 (pDMdop1 contains a D-Dop1 cDNA as a partial EcoR1digest from pcDNAI construct cloned into pBluescrip SK vector). (F. Gotzes et al, Receptors on Channels (1994) 2, 131-141).

The amplification was performed using the following oligonucleotide primers:
5' PCR primer (Dop D1/5 FR);
5' – TTTT AAGCTT AGATCT GCCACC ATG TAC ACA CCA ACA CCC ATTT G – 3'
3' PCR primer (Dop D1/5 RV);
5' – TTTT GC GG CC GC GTC GAC TCA AAT CGC AGACACCTGCTC – 3'

The 5' primer contains the restriction sides HindIII, and BglII and the consensus translation: enhancing sequence (GCC ACC) (M.Kozak, 1987 supra). The 3' primer contains the restriction sites Not 1 and Sal 1. A PCR product was obtained with Pfu Polymerase (Stratagene) utilising the manufacturers protocol.

The following PCR conditions were used: 1 cycle of denaturation at 96°C for 2 min, followed by 35 cycles of [denaturation at 96°C for 1 min, annealing at 57°C and 60°C for 45 and extension at 72°C for 3 min]; followed by a final extension reaction of 10 min at 72°C. The resulting PCR product was cloned into the PCR-script (Amp SK+) vector (stratagene) using the manufacturers protocol. Sequence analysis of a clone, confirmed the presence of a correctly edited insert. This insert was released from the

PCR-Script (AMP SK+) background using BglII and NotI enzymes (Pharmacia Biotech Products) and cloned into pEV3 (Figure 1a).

Cell culture and cell transfection

Murine erythroleukemia C88 "leaky" (Deisseroth A, 1978 supra) were cultured as in Example2.

The expression construct pEV3D-Dop1 and the reporter construct p3XVIP Ryg(P) See Fig 1b) were co-transformed into Mel-C88L cells by electroporation. Prior to transfection, 30µg of pEV3D-Dop1 and 30µg of p3XVIP hyg (P) were linearised respectively at the unique sites Asp 718 and XmnI.

The Asp718 unique site is found upstream of the neomycin cassette and downstream of the dopamine Expression cassette in the pEV3D-Dop1 vector. The XmnI unique site is found in the ampicillin cassette, which is flanked by both the reporter expression cassette and the hygromycin cassette. Transfection into the cell line Mel-C88L was performed by electroporation as described (M. Antoniou, 1991 supra). After transfection, cells were diluted in culture medium to concentrations of about $10^4$, $10^5$ and $2x10^5$ cells per ml and 1ml aliquots transferred to each well of a 24-well tissue culture plate (reference as in example 2). Twenty four hours after the transfection, G418 was added to a concentration of 1 mg/ml in order to select for stable transfectants which would contain either the pEV3 D-Dop1 expression construct on its own or with the reporter vector p3XVIP hyg (P). Individual clones were picked, or pooled to generate populations, 7 to 10 days after the addition of selective medium.

These clones and populations were visually assessed as being dividing vigorously before being passaged into media containing two selection agents: G418 and hygromycin B at respective concentrations of 1mg/ml and 0.8mg/ml. This was done in order to select only for transfectants having stably integrated both vectors: pEV3D-Dop 1 and p3XVIP. hyg(P).

For RNA purification, cells were maintained in exponential growth by passaging them every day for a period of 4 days (cells should be growing from $2x10^5$ cells/ml to $6\text{-}8x10^5$ cells/ml in 24 hours when in log phase). One half of the cells was then induced using 2% DMSO and incubated for a further period of 4 days. RNA analysis was carried out as described in Example 3.

β-galactosidase assays using dopamine clones

6 dopamine clones were washed in phenol red free RPMI media (GIBCO) containing 5% FCS and 1% glutamine and resuspended at concentrations of $1.25x10^7$ cells/ml and $2.5x10^6$ cells/ml. Cells were transferred to 96-well microtitre plates a final concentrations of $2x10^5$ cells/well and $1x10^6$ cells/well. Additions of either

a) Forskolin (3μM) or

b) Dopamine (agonist) 10μM or

c) Media

were made at each of the cell concentrations. 2 replicates/clone/concentrations were set up.

After incubation for 5 hours at 37°C in an atmosphere of 10% $CO_2$, a chlorophenol red β-D-galactopyranoside (CPRG) solution was added. The solution comprised

11.4mg CPRG (Boehringer), 500μl 10 x Z buffer, 75μl 20% SDS, 5ml $H_2O$ and 7μl mercaptoethanol.

10 x Z buffer (0.47M $Na_2HPO_4(2H_2O)$, 0.4M $NaH_2PO_4(2H_2O)$, 0.1M KCl, 10mM $MgSO_4(7H_2O)$, pH7.0 with NaOH.

Plates were incubated overnight under the same conditions and the absorbance at 570nm read using a spectrohotometer. Results for the dopamine clones at a concentration of $1x10^6$ cells/well are shown in Figure 7. Clones C and 2 showed an increase in absorbance at 570nm, indicating an increase in cyclic AMP, both in the presence of forskolin and in the presence of dopamine. This signal may be useful in detecting agonists and antagonists of the dopamine receptor.

The serotonin clones showed a similar increase in cyclic AMP in the presence of forskolin. In the presence of serotonin no effect was seen but in the presence of forskolin and serotonin a decrease in forskolin stimulated cAMP was observed. This signal may be useful in detecting agonists and antagonists of the serotonin receptor.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

Claims

1. An erythroid cell which is substantially undifferentiated but which expresses proteins under the control of a globin promoter thereof.

2. An erythroid cell according to claim 1 which is a murine erythroleukaemia (MEL) cell, rat erythroleukaemia cell (REL) or a human erythroleukaemia cell (HEL).

3. An erythoid cell according to claim 2 which is a murine erythroleukaemia cell.

4. An erythoid cell according to any one of claims 1 to 3 wherein the said globin promoter is the β-globin promoter.

5. An erythoid cell according to any one of the preceding claims which comprises a cell as deposited at the European Collection of Cell Cultures under Accession number 99012801.

6. A method of producing an erythroid cell according to any one of the preceding claims which method comprises maintaining growing uninduced erythroid cells in culture for a sufficient period of time and isolating a subclone which expresses protein under the control of a globin promoter.

7. A method for determining the interaction between a receptor protein and a potential agonist or antagonist therefor, said method comprising incubating a cell as defined above which has been transformed so that it expresses said receptor protein as a G-protein coupled receptor, either

(I) in (a) the presence and (b) the absence of said potential agonist; and/or

(II) in the presence of a known agonist and (a) the presence or (b) the absence of said potential antagonist; and

monitoring and comparing G-protein coupled receptor induced signals in cells of (Ia) and (Ib) and/or (IIa) and (IIb).

8. A method according to claim 7 wherein the G-protein coupled receptor induced signal is monitored by measuring the calcium ion content of the cells.

9. A method according to claim 7 wherein the calcium levels are measured by means of a fluorescent indicator.

10. A method according to claim 7 wherein the G-protein coupled receptor induced signal results in a change in the cyclic AMP (cAMP) levels within the cell, and the G-protein induced signal is monitored by measuring the cyclic AMP content of the cells.

11. A method according to claim 7 wherein the cells are transformed with a reporter gene, expressed of which is regulated by a G-protein coupled receptor induced signalling cascade, and the G-protein coupled receptor induced signal is monitored by detecting the product of the reporter gene.

12. A method according to claim 11 wherein the reporter gene is β-GAL.

13. A method according to any one of claims 7 to 12 wherein the G-protein coupled receptor induced signal results in a decrease in the level of the measured cellular component, and tests (I) and (II) are carried out in the presence of a chemical which stimulates the production of said cellular component.

14. A method according to claim 13 wherein the measured cellular component is cAMP and the said chemical is forskolin.

15. A method according to any one of claims 7 to 14 wherein the receptor is an insect receptor.

16. A method according to claim 15 wherein the insect receptor is a tyramine, a serotonin, a dopamine, an octopamine or a muscarinic -acetylcholine receptor.

17. A method according to any one of claims 7 to 16 wherein the cells are subsequently induced to differentiate, and used in a ligand binding assay.

18. An assay for detecting binding between a protein and a potential binding partner therefore, said method comprising (a) transforming a cell according to claim 1 so that the protein is expressed under the control of a globin promoter, (b) detecting binding between said potential binding partner and the said protein on a membrane of the cell.

19. An assay according to claim 18 where the cells are induced after step (a) and prior to step (b), so as to obtain high levels of protein expression from fully differentiated cells.

20. An assay according to claim 18 or claim 19 wherein step (b) may be effected on isolated membranes extracted from lysed cells.

21. A vector comprising a sequence which encodes a non-mammalian protein receptor under the control of a globin promoter.

22. A vector according to claim 21 wherein the globin promoter is under the control of the human globin locus control region.

23. A vector according to claim 21 or claim 22 wherein the non-mammalian protein receptor is an insect receptor.

24. A vector according to claim 23 wherein the insect receptor is the locust tyramine receptor.

25. A cell according to any one of claims 1 to 5 which is transformed with a vector according to any one of claims 21 to 24.

Abstract

# CELL AND ASSAY

An erythroid cell which is substantially undifferentiated but which expresses proteins under the control of a globin promoter thereof, is described and claimed. Cells of this type are particularly useful in functional assays.

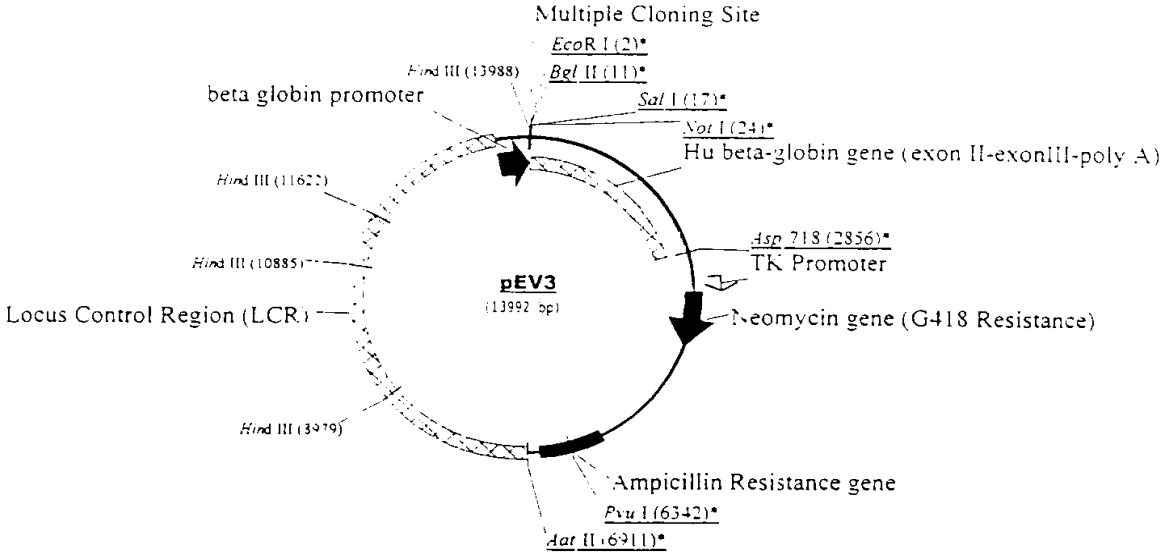



**Figure 1a**

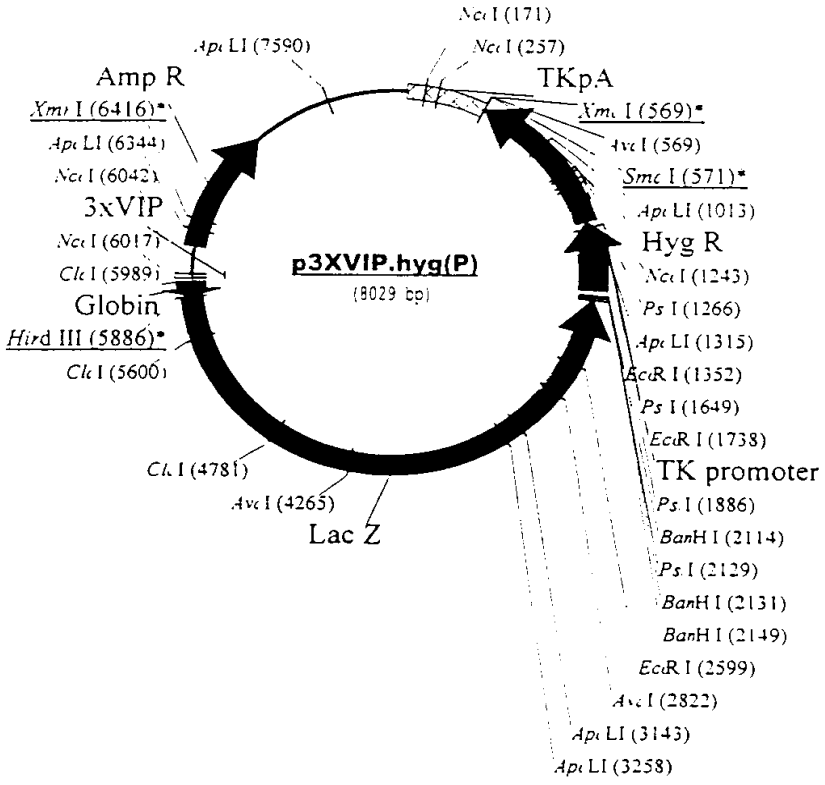
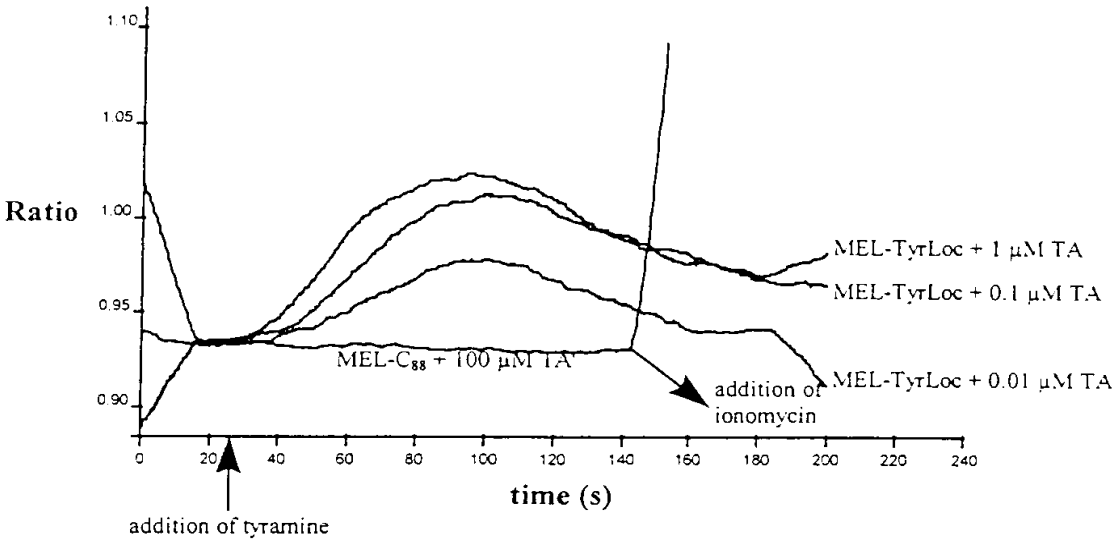



Figure 1b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1.05
1.00
0.95
0.90
0
20
40
60
80
100
120
140
160
180
200
220
240
time (s)
addition of tyramine
addition of ionomycin
MEL-TyrLoc + 1 µM TA
MEL-TyrLoc + 0.1 µM TA
MEL-TyrLoc + 0.01 µM TA
MEL-C88 + 100 µM TA

**Figure 2**

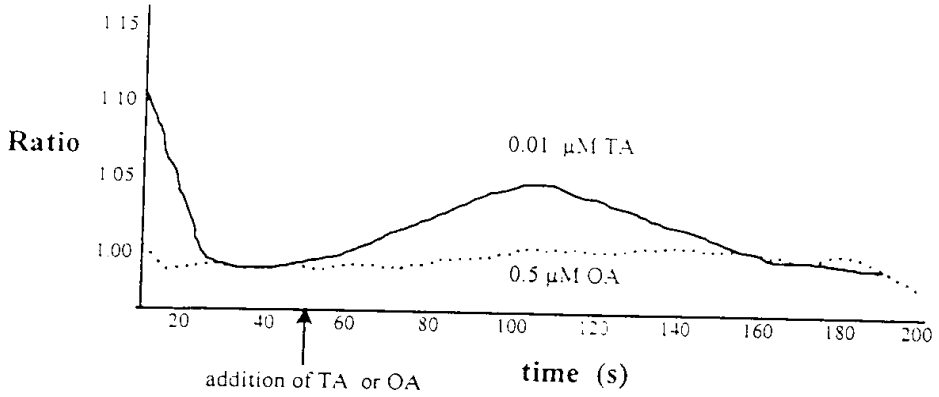


Figure 3

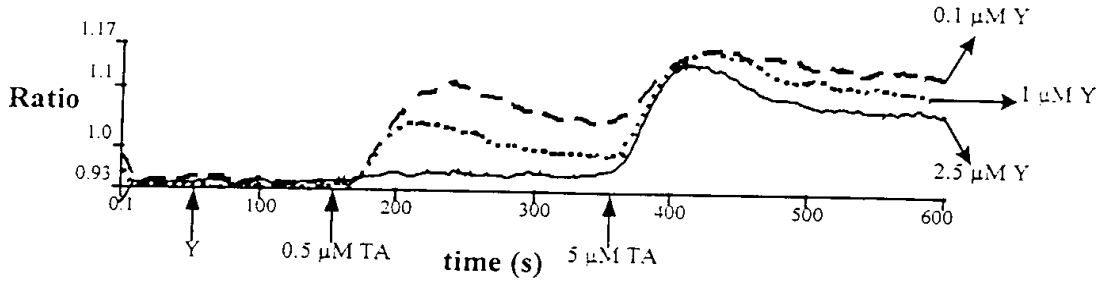


Figure 4

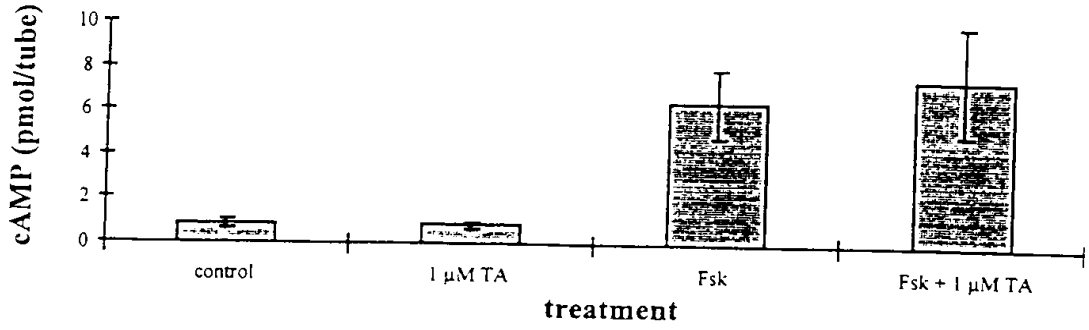


Figure 5

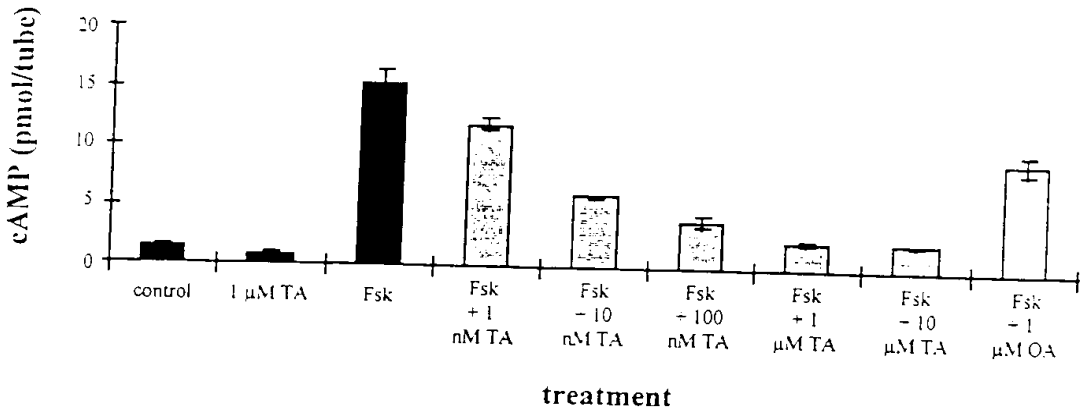


Figure 6

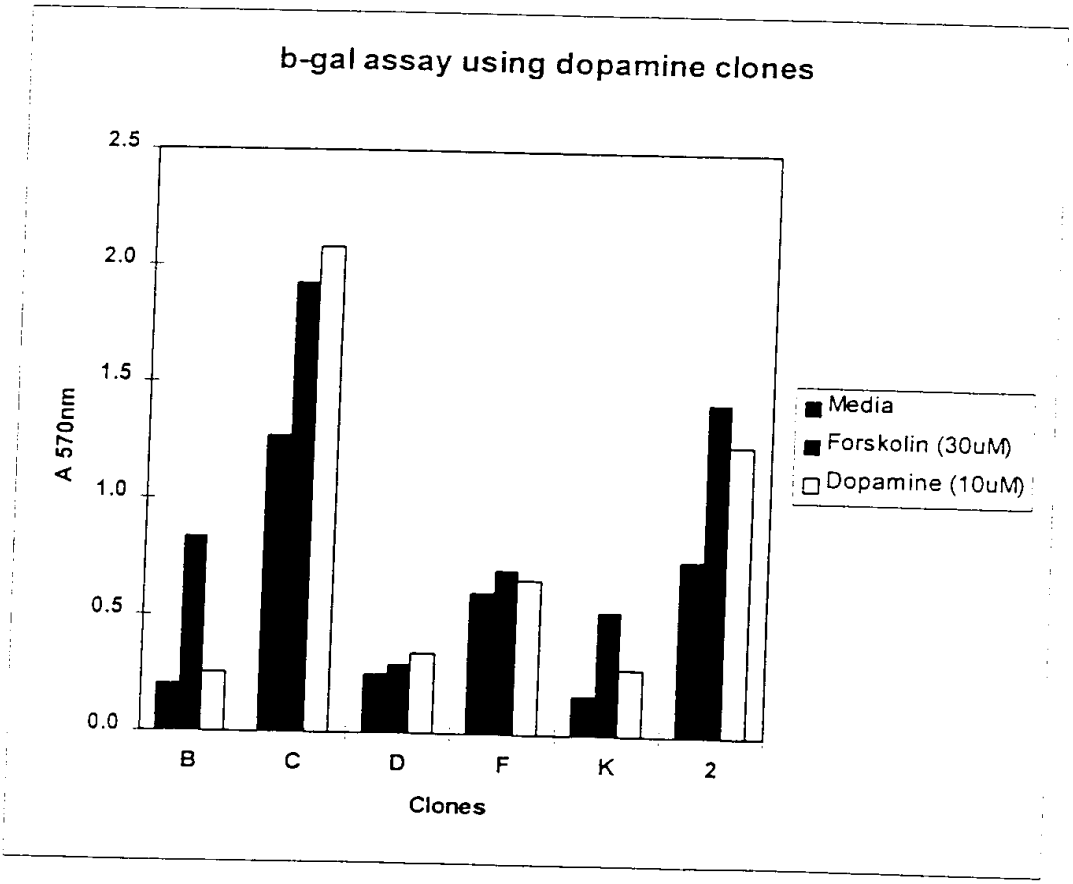


Figure 7

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